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Systematic Evaluation of the Prognostic Impact and Intratumour Heterogeneity of Clear Cell Renal Cell Carcinoma Biomarkers

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Abstract

Background: Candidate biomarkers have been identified for clear cell renal cell carcinoma (ccRCC) patients, but most have not been validated.
Objective: To validate published ccRCC prognostic biomarkers in an independent patient cohort and to assess intratumour heterogeneity (ITH) of the most promising markers to guide biomarker optimisation.
Design, setting, and participants: Cancer-specific survival (CSS) for each of 28 identified genetic or transcriptomic biomarkers was assessed in 350 ccRCC patients. ITH was interrogated in a multiregion biopsy data set of 10 ccRCCs.
Outcome measurements and statistical analysis: Biomarker association with CSS was analysed by univariate and multivariate analyses.
Results and limitations: A total of 17 of 28 biomarkers (*TP53* mutations; amplifications of chromosomes 8q, 12, 20q11.21q13.32, and 20 and deletions of 4p, 9p, 9p21.3p24.1, and 22q; low EDNRB and TSPAN7 expression and six gene expression signatures) were validated as predictors of poor CSS in univariate analysis. Tumour stage and the ccB expression signature were the only independent predictors in multivariate analysis. ITH of the ccB signature was identified in 8 of 10 tumours. Several genetic alterations that were significant in univariate analysis were enriched, and chromosomal instability indices were increased in samples expressing the ccB signature. The study may be underpowered to validate low-prevalence biomarkers.
Conclusions: The ccB signature was the only independent prognostic biomarker. Enrichment of multiple poor prognosis genetic alterations in ccB samples indicated that several events may be required to establish this aggressive phenotype, catalysed in some tumours by chromosomal instability. Multiregion assessment may improve the precision of this biomarker.
Patient summary: We evaluated the ability of published biomarkers to predict the survival of patients with clear cell kidney cancer in an independent patient cohort. Only one molecular test adds prognostic information to routine clinical assessments. This marker showed good and poor prognosis results within most individual cancers. Future biomarkers need to consider variation within tumours to improve accuracy.

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1. Introduction

The clinical behaviour of clear cell renal cell carcinomas (ccRCCs) is highly variable, ranging from slow-growing localised tumours to aggressive metastatic disease. Thus prognostic markers are important to guide therapeutic intervention and follow-up strategies. Prognostic markers in routine clinical use include tumour stage and grade and prognostic models and nomograms that can also incorporate necrosis, blood tests such as lactate dehydrogenase, haemoglobin, platelets, and calcium levels, prior nephrectomy, symptoms, and performance status [1–6]. However, the accuracy of predictions remains limited for individual patients.

Molecular ccRCC characteristics including genetic alterations and gene expression profiles have been identified as potential novel prognostic biomarkers, but most of these have not been independently validated. Even those that have been validated have not entered clinical practice. Neither have these biomarkers been compared with each other to identify lead candidates for further development.

The analysis of multiple tumour regions from individual ccRCCs recently identified substantial intratumour heterogeneity (ITH). Spatially separated subclones harbouring distinct driver mutations and somatic copy number aberrations (SCNAs) were present within primary tumours and between primary tumours and metastases [7–9]. Phylogenetic reconstruction revealed branched evolution, demonstrating that multiple subclones were evolving simultaneously within individual tumours. Assessment of a validated prognostic gene expression signature [10] showed expression of the good prognosis ccA signature or poor prognosis ccB signature in different tumour regions within the same patient [7]. Thus ITH with spatially separated subclones can lead to sampling biases that may contribute to the lack of clinically qualified biomarkers in ccRCC. Such observations raise questions regarding how biomarker discovery strategies can be improved in heterogeneous tumours.

We identified genetic and transcriptomic prognostic biomarkers through a literature search to independently validate them in The Cancer Genome Atlas (TCGA) consortium cohort of 350 ccRCC patients [11]. Independent predictors of cancer-specific survival (CSS) were identified in multivariate analysis, and the impact of ITH was assessed.

2. Methods

2.1. Literature search

Published genetic or transcriptomic prognostic biomarkers for RCC patients were identified in PubMed and Google Scholar. Keywords included *biomarker, prognosis*, and *renal cell carcinoma*. Literature cited in review articles was also assessed. Publications had to be in the English language. Studies exclusively based on non–clear cell histology were excluded. Details of publications excluded for technical reasons can be found in the Supplement.

2.2. Validation cohort

Somatic mutation ($n = 417$) and clinical data ($n = 446$) were obtained from [11]. Single nucleotide polymorphism (SNP) array ($n = 450$) and RNA sequencing (RNA-seq) data ($n = 469$) for the same cohort were downloaded (https://tcga-data.nci.nih.gov/tcga/) on March 14, 2012, and September 18, 2012, respectively. The molecular and clinical data for our analysis were available for 350 of these patients. We used our previously published multiregion gene expression data sets GSE31610 and GSE3000 [7,8] for the assessment of ITH (data sets available at http://www.ncbi.nlm.nih.gov/geo/). Data processing is described in the Supplement.

2.3. Statistical analysis

CSS was assessed by the Kaplan-Meier method from the initial pathologic diagnosis to death with tumour as the end point. Statistical significance was assessed with the log-rank test. Hazard ratios (HRs) were calculated using univariate Cox regression analysis. Competing risk analysis was performed using death with tumour as the end point and death without tumour as the competing risk event. Variables with $p \leq 0.05$ were included into multivariate Cox regression analysis with backwards stepwise selection.

Differences in enrichment of genetic aberrations and genomic instability indices in ccA and ccB subgroups were assessed by the Fisher exact test and the Wilcoxon test, respectively. Details of the statistical analysis are provided in the Supplement.

3. Results

3.1. Identification of prognostic biomarkers

The literature search identified 30 publications describing RCC prognostic genetic or gene expression markers. Three multigene expression signatures with < 70% of probes mapping to genes annotated in the TCGA RNA-seq data set and one signature based on a mathematical model optimised for array expression data and not readily applicable to RNA-seq data were excluded. Overall, 28 candidate biomarkers were identified from the remaining 26 publications for validation (Table 1).

3.2. Biomarker validation by univariate analysis

The median follow-up of the validation cohort was 51 mo. Clinical/pathologic characteristics (Table 2) were similar to the RCC cohorts in which the candidate biomarkers had been identified (Supplemental Table 1). All patients had undergone nephrectomy from which the samples for molecular analysis had been taken. Higher tumour stage and grade were significantly associated with poor CSS (Table 3 and Fig. 1) as expected. Other established clinical prognostic variables such as blood test results, performance status, or necrosis were not available for all patients and were not evaluated. A total of 19 of 28 molecular biomarkers were significantly associated ($p \leq 0.05$) with CSS (Table 3).

3.2.1. Somatic mutations

Mutations in five driver genes were described as potential prognostic markers [11–18], but only nonsynonymous mutations in the BRCA1 associated protein-1 (ubiquitin carboxy-terminal hydrolase) (*BAP1*) (HR: 1.94; $p = 0.022$) and tumour protein 53 (*TP53*) (HR: 5.09; $p < 0.001$) tumour suppressor genes were validated as predictors of poor CSS

Table 1 – Candidate prognostic biomarkers identified in the literature search

Variable	Prognosis	Analysis	Cohort size* (*n*)	Reference
Somatic mutations				
VHL (loss of function+ mutations)	Poor (OS/PFS)	Sequencing	56	Kim et al. [12]
VHL (loss of function+ mutations)	Poor (CSS)	Sequencing	83	Schraml et al. [13]
VHL (somatic mutations)	Better (CSS/CFS)	Sequencing	134	Yao et al. [14]
PBRM1	Better (OS)	Sequencing	145 + 327	Kapur et al. [15]
BAP1	Poor (OS)	Sequencing	145 + 327	Kapur et al. [15]
BAP1	Poor (CSS)	Sequencing	188 + 421	Hakimi et al. [16]
BAP1	Poor (OS)	Sequencing	>400	TCGA consortium [11]
BAP1	Poor (OS)	Sequencing	240	Sato et al. [17]
SETD2	Poor (CSS)	Sequencing	188 + 421	Hakimi et al. [16]
SETD2	Poor (CFS)	Sequencing	240	Sato et al. [17]
TP53	Poor (CSS)	Sequencing	416	Kandoth et al. [18]
Somatic copy number variations				
5q31-qter (5q focal) Amplification	Better (CSS)	Cytogenetics	104	Gunawan et al. [19]
7q36.2 (7q focal) Amplification	Poor (CSS)	Array CGH, FISH	53	Sanjmyatav et al. [20]
8q Amplification	Poor (CSS)	Cytogenetics	336	Klatte et al. [21]
8q Amplification	Poor (OS)	SNP array	85	Monzon et al. [22]
12 Amplification	Poor (RFS)	Cytogenetics	50	Elfving et al. [25]
20q11.21q13.32 (20q focal) Amplification	Poor (CSS)	Array CGH, FISH	53	Sanjmyatav et al. [20]
20 Amplification	Poor (RFS)	Cytogenetics	50	Elfving et al. [25]
3p Deletion	Better (CSS)	Cytogenetics	246	Klatte et al. [23]
3p Deletion	Better (CSS)	Cytogenetics	288	Kroeger et al. [24]
4p Deletion	Poor (CSS)	Cytogenetics	246	Klatte et al. [23]
8p Deletion	Poor (RFS)	Cytogenetics	50	Elfving et al. [25]
9p21.3p24.1 (9p focal) Deletion	Poor (CSS)	CGH, FISH	53	Sanjmyatav et al. [20]
9p Deletion	Poor (CSS)	Cytogenetics	246	Klatte et al. [23]
9p Deletion	Poor (CSS/RFS)	Cytogenetics, FISH	703	La Rochelle et al. [26]
9p Deletion	Poor (RFS)	CGH	37	Moch et al. [27]
9p Deletion	Poor (CSS)	FISH	73	Brunelli et al. [28]
14q Deletion	Poor (CSS)	Cytogenetics	246	Klatte et al. [23]
14q Deletion	Poor (CSS)	Cytogenetics	288	Kroeger et al. [24]
14q Deletion	Poor (OS/RFS)	SNP array	85	Monzon et al. [22]
19 Deletion	Poor (CSS)	Cytogenetics	131	Antonelli et al. [29]
22 Deletion	Poor (CSS)	Cytogenetics	131	Antonelli et al. [29]
Gene expression analysis				
CD31, EDNRB, and TSPAN7 expression levels	Higher expression levels of each are better (CSS)	mRNA arrays	24	Wuttig et al. [30]
Aggressive and nonaggressive ccRCCs classified using 35 genes (26 [74%] genes assessed in current study)	Aggressive worse than nonaggressive (CSS)	mRNA arrays	66	Kosari et al. [31]
Two gene expression clusters classified using 259 genes (220 [85%] genes assessed in current study)	Cluster 2 worse than cluster 1 (CSS)	mRNA arrays	177	Zhao et al. [33]
Indolent and aggressive ccRCC classified using 44 genes (36 [82%] genes assessed in current study)	Aggressive worse than indolent	cDNA arrays	38	Lane et al. [32]

Table 1 – (*Continued*)

Variable	Prognosis	Analysis		
ccA/ccB subgroup classified using 110 genes (103 [94%] genes assessed in current study)	ccB worse than ccA (CSS)	mRNA arrays	48 + 177	Brannon et al. [10]
Cluster A, B, and C classified using 48 (B vs A/C) and 23 (A vs C) genes, respectively (37 [77%] and 21 [91%] genes, respectively, assessed in current study)	Cluster A better than B and C, with C having the poorest prognosis (CSS)	mRNA arrays	176	Beleut et al. [34]
TGFβ signature:scored with a panel of 157 TGFβ genes (145 [92%] genes assessed in current study)	Poor for higher expression (CSS)	mRNA arrays	176	Boström et al. [35]

CSS = cancer-specific survival; mRNA = messenger RNA; OS = overall survival; PFS = progression-free survival; SNP = single nucleotide polymorphism; TGF = tumour growth factor.
* The cohort size in this table signifies the number of cases for which follow-up data was available.
+ Loss of function mutation was defined as frameshift or nonsense mutations.

(Fig. 1 and Table 3). CSS was not significantly different for patients with nonsynonymous mutations in polybromo 1 (*PBRM1*), SET domain containing 2 (*SETD2*), or von Hippel-Lindau tumour suppressor, E3 ubiquitin protein ligase (*VHL*) (Supplemental Fig. 1). Restricting the analysis to *VHL* loss-of-function mutations (frameshift and nonsense mutations) in accordance with Kim et al. [12] and Schraml et al. [13] or to stage I–III cases only [14] did not change the results.

Table 2 – Patient and tumour characteristics of the validation cohort

Variable	TCGA cohort (n = 350)
Age, yr	
Median (IQR)	61 (52–70)
Gender (%)	
Male	222 (63)
Female	128 (37)
Fuhrman grade (%)	
G1	4 (1)
G2	145 (41)
G3	146 (42)
G4	55 (16)
Clinical stage (%)	
Stage I	162 (46)
Stage II	34 (10)
Stage III	96 (27)
Stage IV	58 (17)
Primary tumour spread (%)	
T1	166 (48)
T2	40 (11)
T3	139 (40)
T4	5 (1)
Metastatic spread (%)	
M0	293 (84)
M1	57 (16)
Lymph node spread (%)	
N0	168 (48)
N1	8 (2)
NX (Undetermined)	174 (50)
Median follow-up	51 mo
Total no. of deaths	121
No. of deaths from ccRCC	80

ccRCC = clear cell renal cell carcinoma; TCGA = The Cancer Genome Atlas.

3.2.2. *Somatic copy number alterations*

Four focal SCNAs [19,20], six arm-level alterations [21–28], and four whole chromosome alterations [25,29] have been identified as candidate biomarkers. Several of these SCNAs have been identified by cytogenetic and other low-resolution analyses. Copy number profiles generated from high-resolution SNP array data from TCGA was converted into lower resolution cytoband-level data to facilitate comparison. Amplification or deletion of ≥50% of a chromosome arm or of both arms of a chromosome was considered to be equivalent to an arm-level alteration as described [11] or to a whole chromosome aberration, respectively.

Nine of 14 unique SCNAs were validated as poor prognostic markers. Chromosome 8q (Chrom8q) amplification (HR: 2.70; $p < 0.001$), Chrom12 amplification (HR: 1.74; $p = 0.034$), Chrom20 focal amplification (HR: 2.44; $p < 0.001$), Chrom20 amplification (HR: 2.37; $p < 0.001$), Chrom4p deletion (HR: 1.97; $p = 0.019$), Chrom9p focal deletion (HR: 2.33; $p < 0.001$), Chrom9p deletion (HR: 2.56; $p < 0.001$), Chrom19 deletion (HR 3.25; $p = 0.034$), and Chrom22q deletion (HR: 2.23; $p = 0.012$) were significantly associated with poor CSS (Fig. 1 and Table 3). The remaining five SCNA markers failed validation (Supplemental Fig. 1).

3.2.3. *Gene expression analysis*

EDNRB and TSPAN7 gene expression above defined cut-offs [30] correlated with better CSS (HR: 0.37; $p < 0.001$ and HR: 0.29; $p < 0.001$, respectively), but CD31 overexpression was not significant. Non-negative matrix factorisation (NMF) clustering was applied for each multigene expression signature [10,31–34] to identify samples with distinct expression profiles (Supplemental Fig. 2). All prognostic

Table 3 – Univariate survival analysis

Variable	No. of cases (n = 350) (%)	HR (95% CI)	p value
Clinical and pathologic characteristics			
Stage II vs stage I	34 (10)	4.45 (1.55–12.77)	0.006
Stage III vs stage I	96 (27)	7.34 (3.16–17.08)	<0.001
Stage IV vs stage I	58 (17)	25.24 (11.26–56.71)	<0.001
G3 vs G1/G2	146 (42)	2.35 (1.30–4.26)	0.005
G4 vs G1/G2	55 (16)	7.43 (3.99–13.81)	<0.001
Somatic mutations			
VHL loss of function mutation	86 (24.5)	0.59 (0.34–1.04)	0.064
VHL nonsyn mutation (all cases)	178 (51)	0.80 (0.51–1.25)	0.323
VHL nonsyn mutations (stage I–III cases)	155/292 (53)	0.95 (0.50–1.80)	0.873
PBRM1 nonsyn mutation	117 (33)	0.90 (0.56–1.43)	0.643
BAP1 nonsyn mutation	37 (10.5)	1.94 (1.08–3.45)	0.022
SETD2 nonsyn mutation	39 (11)	1.41 (0.76–2.60)	0.273
TP53 nonsyn mutation	7 (2)	5.09 (1.85–14.00)	<0.001
Somatic copy number variations			
5q focal amplification	191 (54.5)	0.72 (0.47–1.12)	0.143
7q focal amplification	95 (27)	1.29 (0.81–2.05)	0.283
8q amplification	33 (9)	2.70 (1.52–4.81)	<0.001
12 amplification	56 (16)	1.74 (1.04–2.91)	0.034
20q focal amplification	51 (15)	2.44 (1.49–3.99)	<0.001
20 amplification	47 (13)	2.37 (1.41–3.97)	<0.001
3p deletion	318 (91)	0.86 (0.41–1.79)	0.687
4p deletion	42 (12)	1.97 (1.10–3.52)	0.019
8p deletion	101 (29)	1.58 (0.99–2.50)	0.051
9p focal deletion	85 (24)	2.33 (1.49–3.64)	<0.001
9p deletion	88 (25)	2.56 (1.64–3.99)	<0.001
14q deletion	140 (40)	1.51 (0.97–2.35)	0.064
19 deletion	6 (1.7)	3.25 (1.02–10.32)	0.034
22q deletion	26 (7)	2.23 (1.18–4.23)	0.012
Gene expression analysis			
CD31 expression			
< median	175 (50)	0.64 (0.41–1.01)	0.051
≥ median	175 (50)		
EDNRB expression			
< median	175 (50)	0.37 (0.23–0.59)	<0.001
≥ median	175 (50)		
TSPAN7 expression			
<33 percentile	105 (30)	0.29 (0.18–0.45)	<0.001
≥33 percentile	245 (70)		
Kosari signature			
Nonaggressive	242 (69)	2.85 (1.84–4.43)	<0.001
Aggressive	108 (31)		
Zhao signature			
Cluster 1 (good)	269 (77)	5.26 (3.37–8.22)	
Cluster 2 (poor)	81 (23)		<0.001
Lane signature			
Indolent	219 (63)	4.21 (2.62–6.77)	<0.001
Aggressive	131 (37)		
ccA/ccB status			
ccA	240 (69)	4.90 (3.09–7.76)	<0.001
ccB	110 (31)		
Beulet signature			
Cluster A	127 (36)	1.00 (Ref)	0.005
Cluster B	175 (50)	2.27 (1.31–3.96)	
Cluster C	48 (14)	2.30 (1.13–4.66)	
TGFβ signature			
Low expression score	175 (50)	1.98 (1.23–3.16)	0.003
High expression score	175 (50)		

CI = confidence interval; HR = hazard ratio; nonsyn = nonsynonymous; TGF = tumour growth factor.

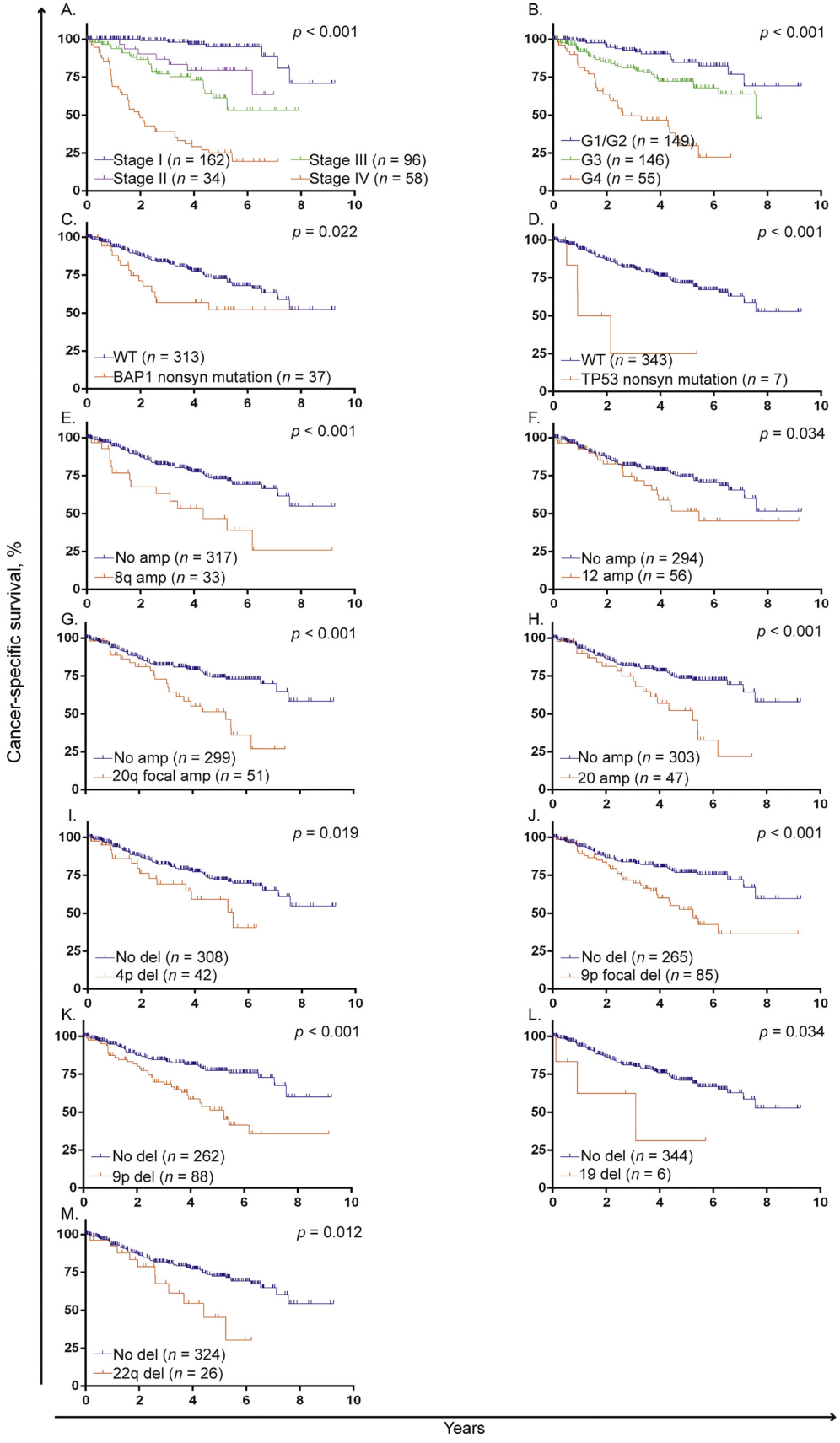

Fig. 1 – Kaplan-Meier survival estimates for cancer-specific survival for clinical and genetic markers: (A) tumour stage; (B) Fuhrman grade; (C) *BAP1* nonsynonymous (nonsyn) mutation status; (D) *TP53* nonsyn mutation status; (E) chromosome (Chrom) 8q amplification (amp) status; (F) Chrom12 amp status; (G) Chrom20q focal amp status; (H) Chrom20 amp status; (I) Chrom4p deletion (del) status; (J) Chrom9p focal del status; (K) Chrom9p del status; (L) Chrom19 del status; (M) Chrom22q del status.
WT = wild type.

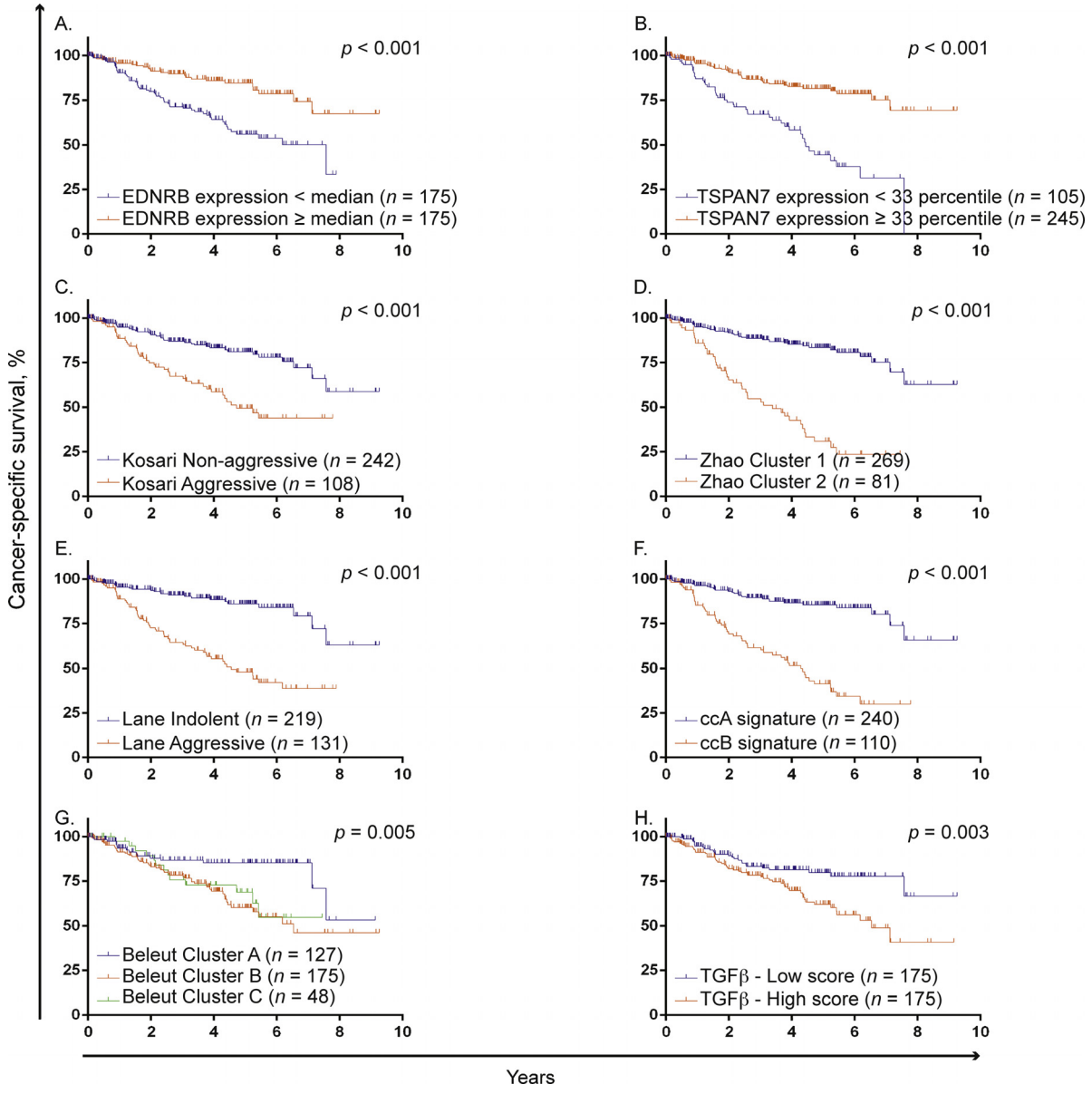

Fig. 2 – Kaplan-Meier survival estimates for cancer-specific survival for gene expression markers: (A) EDNRB expression levels; (B) TSPAN7 expression levels; (C) gene expression subgroup of patients, Kosari signature; (D) gene expression subgroup of patients, Zhao signature; (E) gene expression subgroup of patients, Lane signature; (F) gene expression subgroup of patients, ccA/ccB; (G) gene expression subgroup of patients, Beleut signature; (H) gene expression subgroup of patients according to tumour growth factor (TGF) β activity score.

gene expression signatures validated: the aggressive subgroup defined by Kosari [31] had worse CSS than the nonaggressive subgroup (HR: 2.85; $p < 0.001$); the Zhao [33] poor prognosis cluster 2 had worse CSS than cluster 1 (HR: 5.26; $p < 0.001$). The aggressive subgroup defined by Lane et al. [32] showed worse CSS than the indolent subgroup (HR: 4.21; $p < 0.001$); the Brannon [10] poor prognosis ccB subgroup (HR: 4.90; $p < 0.001$) had worse CSS than the ccA subgroup. Based on Beleut et al. [34], CSS was significantly worse for patients in the poor prognosis clusters C (HR: 2.21; $p = 0.034$) and B (HR: 2.46; $p = 0.002$) than for those in cluster A, although CSS of clusters B and C showed no significant difference. The poor-risk subgroup of Böstrom et al. [35] with a high tumour growth factor β (TGF-β) score had worse CSS than the subgroup with a low score (HR: 1.98; $p = 0.003$) (Fig. 2 and Table 3).

With the exception of *BAP1* mutations and Chrom19 deletions, all markers that were significant in log-rank analysis were also significant in a competing risk analysis including death from causes other than cancer.

3.3. *Identification of independent biomarkers in multivariate analysis*

Chrom9p focal deletion and Chrom20 whole arm amplification were excluded because their HRs were lower than the overlapping Chrom9p arm-level deletions and Chrom20 focal amplifications. The remaining 17 biomarkers that had

Table 4 – Multivariate survival analysis

Variable	Including *BAP1* mutations and chromosome 19 deletion		Excluding *BAP1* mutations and chromosome 19 deletion	
	Hazard ratio (95% CI)	*p* value	Hazard ratio (95% CI)	*p* value
Tumour stage				
Stage I	1.00 (Ref)		1.00 (Ref)	
Stage II	3.48 (1.20–10.06)	0.022	3.40 (1.18–9.82)	0.024
Stage III	4.61 (1.93–11.00)	<0.001	4.86 (2.05–11.55)	<0.001
Stage IV	18.01 (7.89–41.12)	<0.001	17.77 (7.79–40.53)	<0.001
Chromosome 19 deletion	4.18 (1.27–13.69)	0.018	–	–
ccA status	1.00 (Ref)		1.00 (Ref)	
ccB status	2.99 (1.87–4.80)	<0.001	2.95 (1.84–4.72)	<0.001

CI = confidence interval.

been validated in the log-rank analysis were included together with tumour stage and grade into the multivariate analysis (MVA). Tumour stage, the ccA/ccB gene expression signature, and Chrom19 deletions were the only independent predictors of CSS (Table 4 and Supplemental Table 2). After exclusion of the two markers (*BAP1* mutations, Chrom19 deletions) that were not significant in the competing risk analysis, only tumour stage and the ccA/ccB signature remained significant in MVA (Table 4). Based on these results and the small number of six tumours showing Chrom19 deletions, the ccB signature was the lead candidate for further assessment.

The ccB signature was consistently associated with a worse prognosis in patients with stage I (HR > 10; $p < 0.001$), stage II/III (HR: 3.03; $p = 0.003$), and stage IV ccRCCs (HR: 2.15; $p = 0.015$) (Supplemental Fig. 3). A total of 135 patients with stage I tumours expressing the ccA signature demonstrated particularly good outcomes with no cancer-specific deaths for >6 yr. The ccA/ccB signature was also significant in MVA when assessed together with the validated and widely used size, stage, grade, and necrosis (SSIGN) prognostic scoring system [4,36,37] (data available for a subgroup of 334 patients; Supplemental Table 3). CSS of patients whose tumours displayed the ccA or ccB signature were significantly different in three of five validated SSIGN score categories [36,37] (Supplemental Fig. 4). The ccA/ccB signature could not be compared with other clinical nomograms [1,2,5,6] because essential parameters were not available for most of the patients in the TCGA cohort. After completion of our literature search, the ClearCode34 prognostic expression signature was published that is based on the ccA/ccB signature [38]. This signature was significant in univariate analysis and together with tumour stage in MVA if the ccA/ccB signature was omitted (Supplemental Table 4). Although the HR for ClearCode34 in the MVA was lower (HR: 2.23) than that of the ccA/ccB signature (HR: 2.95), the implementation of this 34-gene signature may be easier in clinical practice than the 110-gene ccA/ccB signature.

3.4. Molecular drivers of the ccB subgroup

We next investigated whether the ccB expression signature might reflect the transcriptomic impact of the poor-risk genetic alterations that were significant in log-rank analysis but failed in the multivariate analysis. Seven of nine poor prognosis genetic alterations (*BAP1* and *TP53* mutations; Chrom8q, Chrom12, and Chrom20q focal amplifications; Chrom9p and Chrom22q deletions) were significantly enriched ($p < 0.05$) in the ccB subgroup (Fig. 3). Overall, 72% of the ccB samples showed at least one of these seven aberrations compared with only 30% of ccA samples (Fig. 4A). Both the maximum and the median number of these aberrations per sample were higher in the ccB group than in the ccA group (Fig. 4A and 4B). In contrast, only two of the eight candidate genetic markers that had failed univariate validation were enriched in ccB samples (Supplemental Fig. 5), and the median number of these aberrations between ccA and ccB samples was not statistically different (Fig. 4C and 4D).

Chromosomal instability fosters the acquisition of SCNAs and has been associated with poor prognosis in several cancers [39]. To reveal whether enrichment of chromosomal aberrations in ccB was a result of increased chromosomal instability, we calculated the weighted Genomic Instability Index (wGII), which is a measure of overall copy number aberrations (wGII ≥0.2 is considered unstable [40]). The ccB samples had significantly higher wGIIs compared with ccA samples ($p < 0.001$; Fig. 4E). Based on these results, it appears possible that the aggressive ccB phenotype is partially driven by several poor prognosis SCNAs co-occurring within these samples, permitted by a cancer genomic background of elevated chromosomal instability.

3.5. Intratumour heterogeneity of the ccA/ccB signature

We previously found that the ccA and the ccB signatures were present simultaneously within an individual ccRCC [7]. To investigate whether this signature commonly displays ITH, we reanalysed our published gene expression data of 63 tumour regions from 10 stage II–IV ccRCCs [7,8] (Supplemental Fig. 6) and mapped the results onto the phylogenetic trees previously published for these tumours [8] (Fig. 5). Only two tumours homogeneously expressed the ccA signature; the other eight tumours were heterogeneous with ccA and ccB components detectable, suggesting the need to sample multiple tumour regions to reliably detect poor prognostic clones.

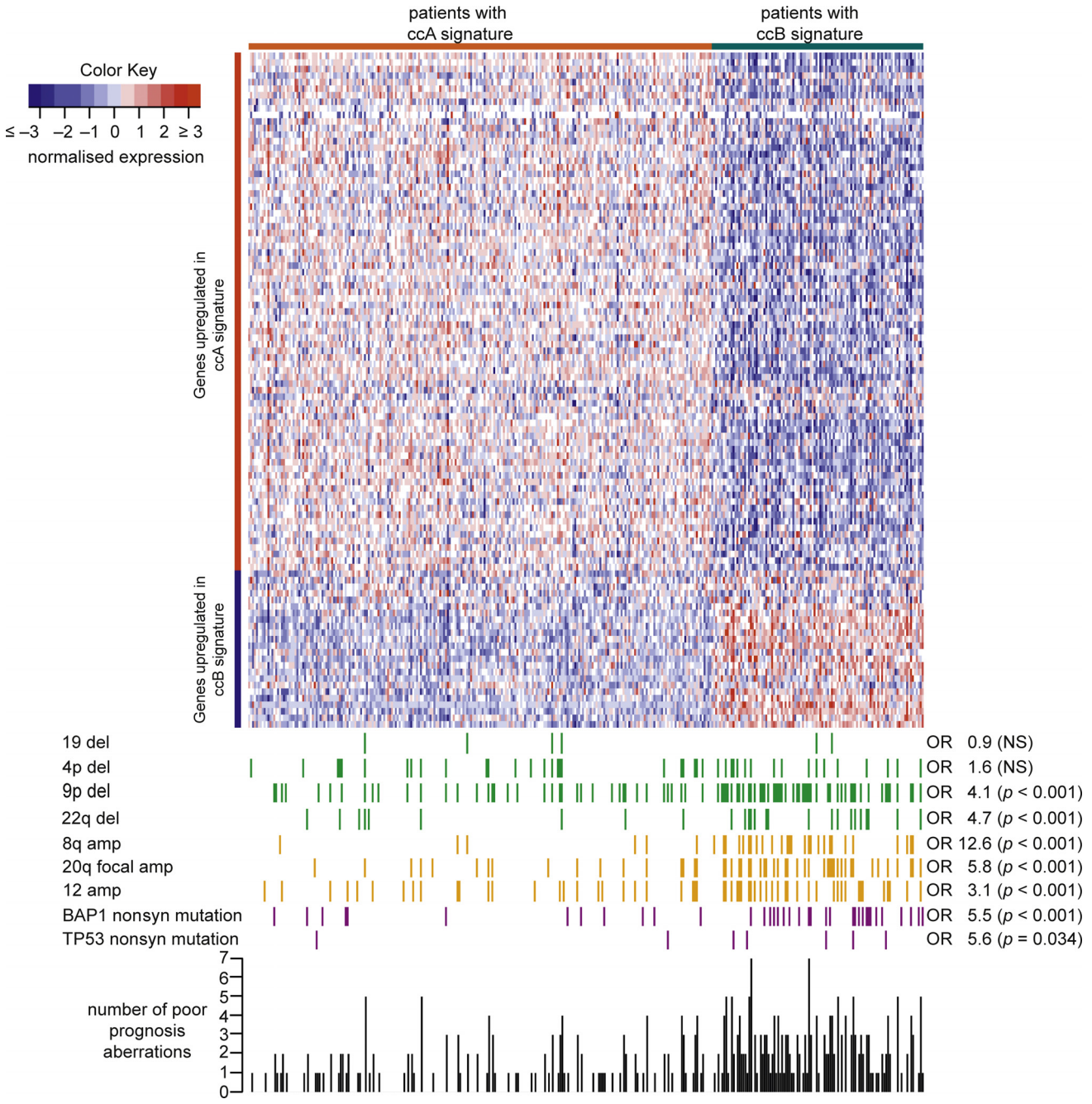

Fig. 3 – Heat map showing consensus non-negative matrix factorisation clustering analysis based on gene expression data of 103 ccA/ccB signature genes. Patient assignment to ccA and ccB prognostic subgroups is indicated by coloured bars at the top of the heat map. Coloured bars below the heat map depict the presence of poor prognosis genetic aberrations. The bar chart at the bottom of the figure represents the number of these genetic aberrations per patient.
OR = odds ratio.

4. Discussion

A total of 17 of the 28 published genetic and transcriptomic prognostic ccRCC markers were validated in log-rank and competing risk analysis as predictors of CSS in this independent validation cohort. Of those, only the ccB gene expression signature was significant in MVA. Tumour stage was the only other independent predictor of CSS in MVA. Importantly, the ccA signature identified patients with stage I ccRCCs who had an excellent prognosis with no cancer-specific deaths over >6 yr of follow-up. The ccA/ccB signature was also significant in MVA with the established SSIGN prediction model, demonstrating that this molecular marker can add additional information to one of the best currently available predictors based on clinical and pathologic information. Thus the ccA/ccB signature could refine personalised follow-up strategies or stratification into adjuvant therapy trials. The novel ClearCode34

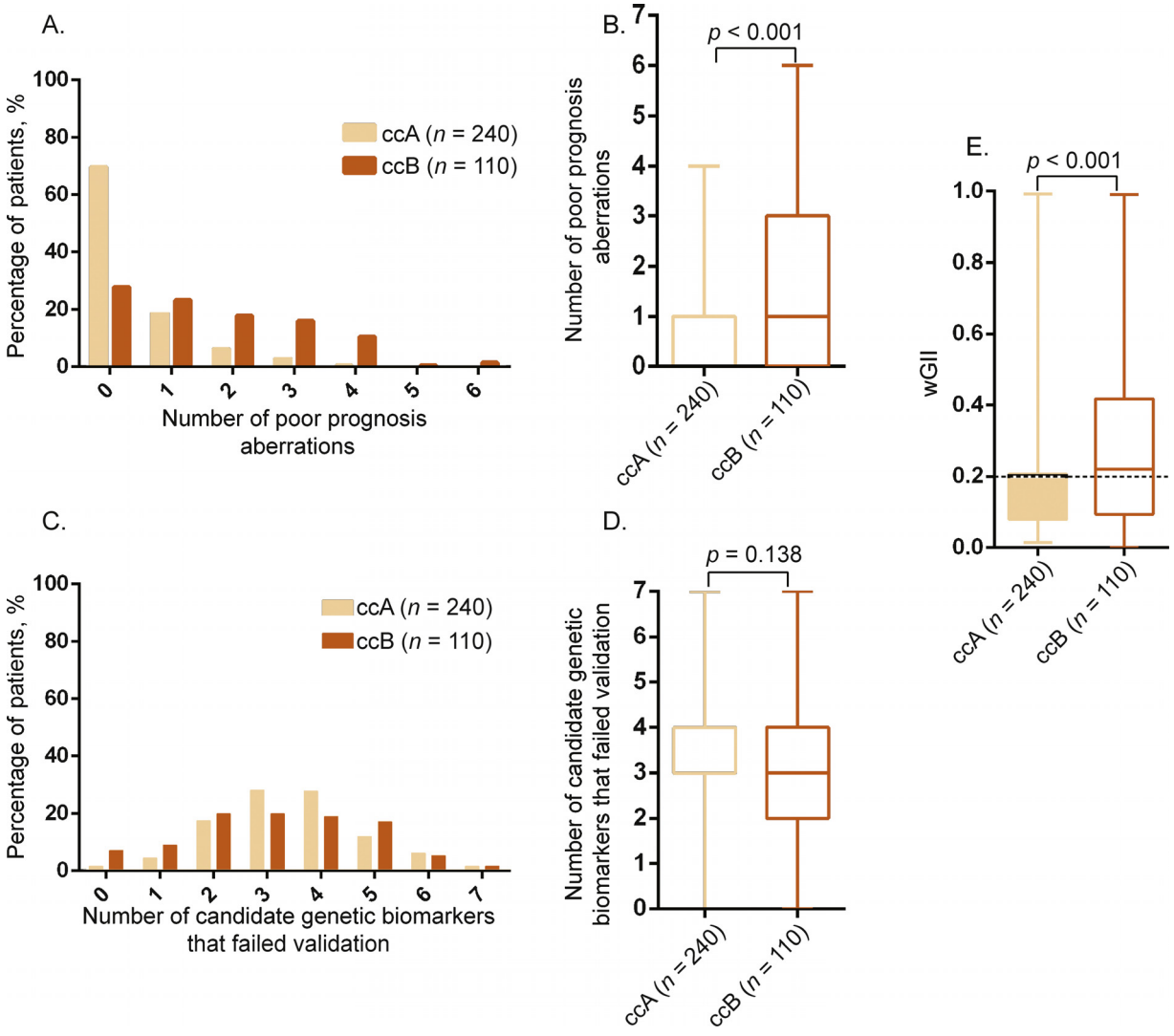

Fig. 4 – (A) Comparison of the number of poor prognosis genetic aberrations per sample between ccA and ccB subgroups. Only aberrations that are enriched in the ccB subgroup were considered. (B) Box and whisker plot comparing median number of poor prognosis genetic aberrations between samples assigned to the ccA and the ccB group. (Wilcoxon test; $p < 0.001$). (C) Comparison of the number of genetic aberrations that did not pass univariate validation per sample between ccA and ccB subgroups. (D) Box plot and whisker plot showing the median number of genetic aberrations that did not pass univariate validation between ccA and ccB subgroups (Wilcoxon test; $p = 0.138$). (E) Box plot and whisker plot comparing weighted Genomic Instability Index (wGII) between ccA and ccB subgroups. wGII ≥0.2 is considered genomically unstable.

signature is based on the ccA/ccB signature but can be assessed from 34 instead of 110 genes. The performance of this new marker was slightly inferior, but it may nevertheless be valuable because clinical adoption may be easier.

Previous work revealed that genes overexpressed in samples with the ccA signature are enriched for genes implicated in angiogenesis and fatty acid, organic acid, and pyruvate metabolism. Genes overexpressed in samples displaying the ccB signature are enriched for cell differentiation, epithelial to mesenchymal transition, mitotic cell cycle, response to wounding, and TGF-β and Wnt signalling genes [10]. We further revealed that seven of nine specific genetic alterations that were validated in univariate analysis were enriched in ccB samples with 72% of samples harbouring at least one and up to six of these. These genetic changes were only found in 30% of the ccA samples with a maximum of four aberrations per sample. Thus the ccB signature may reflect the transcriptomic impact of these poor prognosis alterations, but more than one alteration may be necessary to establish this phenotype, and as yet unknown alterations are also likely to contribute. Arguably, prognostic markers are of limited clinical utility in ccRCC due to the current absence of effective adjuvant strategies. However, further study of the interplay of these genetic aberrations and the pathways deregulated in the ccB signature are clearly necessary to reveal the mechanisms and biologic implications of the ccB phenotype. Such insights could eventually foster the development of specific therapeutic approaches for poor prognosis ccRCC.

Chromosomal instability indices (wGII) were higher in ccB than in ccA samples, suggesting that chromosomal instability may catalyse the evolution of the ccB phenotype

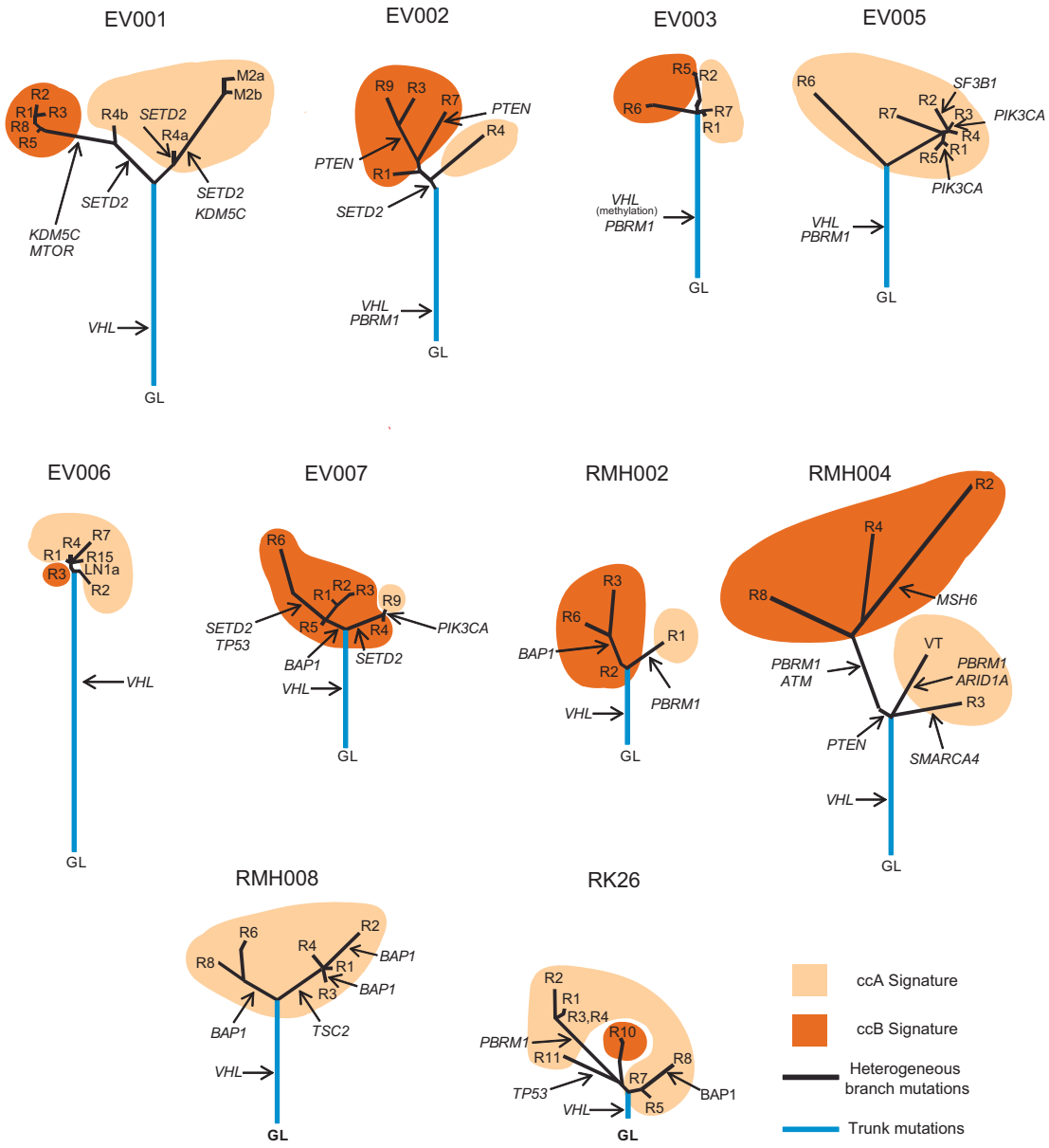

Fig. 5 – Heterogeneity analysis of ccA/ccB expression profiles. The ccA or ccB profiles detected by consensus non-negative matrix factorisation clustering in a multiregion analysis data set from 10 clear cell renal cell carcinomas were mapped onto the phylogenetic trees of these tumours (adapted with permission from Nature Publishing Group [8]). Regional gene expression signatures were assigned to the dominant clones detected within the region. The minority clones detected in some regions in the original publication were omitted.

by providing the permissive heterogeneous genomic background from which these SCNAs can be selected. These results are hypothesis generating and will require further study.

Evaluation of the ccA/ccB signature across multiple tumour regions from each of 10 stage II–IV ccRCCs demonstrated heterogeneous expression patterns with ccA and ccB signatures coexisting in 8 of 10 cases. ITH with spatial separation of subclones that may harbour distinct transcriptomic profiles demonstrates that single biopsies are unlikely to reveal a complete picture of the landscape of even the best current binary classification ccRCC biomarkers.

These data suggest some interesting avenues for research. Despite ITH, the ccB signature outperforms every other candidate biomarker in this analysis. It is currently unknown whether a tumour with a small ccB component has a similarly poor prognosis to an identical size tumour dominated by the ccB signature. If the absolute size of the poor-risk clone, irrespective of the entire tumour population, is the most critical parameter, then ITH may be less problematic in small tumours because the chance of analytical techniques sampling the high-risk cell population would be high. However, detection of a poor-risk ccB clone in larger tumours may be more difficult unless the entire tumour is sampled or dominated by the ccB signature. These

considerations demonstrate that insights into the impact of ITH on clinical outcomes are limited, raising important questions regarding the clinical interpretation of subclonal abundance and how heterogeneous tumours can be better profiled for biomarker discovery and precision medicine.

Several candidate markers that failed validation in univariate and multivariate analyses such as Chrom19 deletion, Chrom8q amplification, and *BAP1* and *TP53* mutations had low prevalence $\leq$10%. This study is underpowered to assess the role of these markers definitively. A further limitation is the lack of protein expression data for the validation cohort that precluded the inclusion of many candidate biomarkers based on immunohistochemistry.

5. Conclusions

Taken together, this study suggests that the ccA/ccB gene expression signature outperforms other transcriptomic and genetic biomarkers for the prediction of ccRCC CSS and that it adds prognostic information to tumour stage and to the SSIGN prognostic model. This signature could be particularly relevant for the profiling of stage I ccRCCs where the detection of the ccA signature was associated with an excellent prognosis. Stage I ccA tumours may only require minimal follow-up, whereas ccB tumours may benefit from more stringent surveillance and may be good candidates for adjuvant therapy trials. Multiregion profiling of larger cohorts could define how to integrate heterogeneity assessments into biomarker predictions and further improve the accuracy of the ccA/ccB signature.

Author contributions: Charles Swanton and Paul A Bates had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Analysis and interpretation of data: Gulati, Gerlinger, Bates, Swanton.
Drafting of the manuscript: Gulati, Gerlinger.
Critical revision of the manuscript for important intellectual content: Gerlinger, Swanton, Gulati, Gore, Larkin, Pickering, Bates, Birkbak, Joshi.
Statistical analysis: Gulati, Bates, Joshi, Birkbak, Szallasi.
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.eururo.2014.06.053.

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